## AD NUMBER

**ADB285703**

## NEW LIMITATION CHANGE

TO

**Approved for public release, distribution unlimited**

FROM

**Distribution authorized to U.S. Gov't. agencies only; Proprietary Information; Jul 2002. Other requests shall be referred to USAMRMC, Ft. Detrick, MD 21702**

## AUTHORITY

**USAMRMC ltr, dtd 10 Jun 2003**

AD_____

Award Number: DAMD17-01-1-0617

TITLE: Is the 66-kDa Isoform of the Shc Adapter Protein a Tumor Suppressor for Growth-Factor-Receptor-Dependent Breast Cancers?

PRINCIPAL INVESTIGATOR: A. Raymond Frackelton, Jr., Ph.D.
Lisa Williams (Nelson)
Pamela Davol

CONTRACTING ORGANIZATION: Roger Williams Hospital
Providence, Rhode Island 02908

REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

## NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

## LIMITED RIGHTS LEGEND

Award Number: DAMD17-01-1-0617
Organization: Roger Williams Hospital

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Carole B. Christian _____

12/4/02 _____

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | July 2002 | Final (1 Jul 01 - 30 Jun 02) |

**4. TITLE AND SUBTITLE**
Is the 66-kDa Isoform of the Shc Adapter Protein a Tumor Suppressor for Growth-Factor-Receptor-Dependent Breast Cancers?

**5. FUNDING NUMBERS**
DAMD17-01-1-0617

**6. AUTHOR(S)**
A. Raymond Frackelton, Jr., Ph.D.
Lisa Williams (Nelson)
Pamela Davol

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Roger Williams Hospital
Providence, Rhode Island 02908

E-Mail: A_Frackelton_Jr@brown.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**



**11. SUPPLEMENTARY NOTES**
Report contains color

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Distribution authorized to U.S. Government agencies only (proprietary information, Jul 02). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 Words)*

In contrast to its 46- and 52-kDa isoforms, the 66-kDa Shc protein functions as a feedback inhibitor of growth-factor signaling and as an apoptotic sensitizer to oxidative stress. Interestingly, p66 Shc is absent or expressed at reduced levels in most breast cancer cell lines.

We have begun to explore the regulation of the p66 Shc isoform expression and to determine the role of its reduced expression in the neoplastic phenotype. Pulse-chase $^{35}$S-Met metabolic labeling and semi-quantitative PCR studies suggest that the reduced expression of p66 Shc is due to regulation at the level of mRNA stability or gene transcription. Forced re-expression of p66-Shc inhibited the ability of breast cancer cell lines to form colonies on soft agar, an *in vitro* correlate of tumorgenicity. Re-expressed p66 Shc appears to be phosphorylated on tyrosine(Y317) and on serine(S36) residues, to be complexed with Grb-2 (29-kDa), and proteins of 22-, 28-, 45-, 50-, 85-, and 105-kDa proteins. The re-expressed p66 Shc does not appear to lower activity of either Erk-1, Erk-2 or phosphatidyl-inositol 3-kinase.

The ability of re-expressed p66 Shc to inhibit anchorage-independent growth indicates that the 66-kDa Shc isoform may act as a tumor suppressor for many breast cancers.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| breast cancer, cell lines, Shc, p66-Shc, tumor suppressor, anchorage-independent growth | 13 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

## INTRODUCTION

Considerable evidence suggests that poor prognosis in breast cancer may be due to overly-activated growth-factor receptors such as ErbB2, the IGF-1 and epidermal growth-factor receptors, and c-Met (see ref 1 for review). A downstream signaling protein common to all these kinases is the adapter protein, Shc. Shc helps activated growth-factor receptors to transfer signals to the c-Ras—MAP kinase pathway (and thus facilitates passage through the G1 phase of the cell cycle...requisite for DNA synthesis and cell proliferation). Additionally Shc appears to signal to Jun kinase and to Myc (implicated in cell proliferation, apoptosis (programmed cell death) , and cellular responses to stress). Recently, we have shown that many human breast cancer cell lines (but not non-cancerous breast epithelial cell lines) not only harbor constitutively activated (phosphorylated on tyrosine 317) Shc proteins (the 52-kDa and 46-kDa isoforms)(see ref. 2, below), but the breast cancer cells require signaling from Shc in order to proliferate(ref. 3).

A third, 66-kDa isoform of Shc(p66 Shc) can act as a feedback inhibitor of growth-factor signaling to MAP kinase and to c-fos(ref 4,5), and as an apoptotic sensitizer for oxidative stress(ref 5,6). Previously we have reported a strong negative correlation ($r=-0.94$, $p<0.001$) between the cellular levels of activated Shc and cellular expression of p66 Shc in the breast cancer lines.

For the breast cancer cells that depend upon constant signaling from p52/46 Shc, and that thereby are under constant oxidative stress, we hypothesized that there would be strong selective pressures to reduce the level of expression (or function) of the 66-kDa Shc isoform. Thus p66 Shc may function as a novel tumor suppressor for these breast cancers.

We began testing this novel hypothesis by forcing these breast cancer cells to re-express normal levels of p66 Shc, and then determining the effect of p66 Shc re-expression on signaling pathways and on the neoplastic phenotype.

Specific objectives were to determine the effects of p66 Shc re-expression on:

i) constitutive and growth-factor-induced signaling to MAP kinases, c-fos, Jun kinase and Myc. Inasmuch as p66 Shc reportedly acts as a feed-back down-regulator of growth factor-receptor signaling, we would expect that p66 Shc re-expression might reduce constitutive signaling to MAPK, c-fos, and perhaps c-Myc. However, insofar as the Jun kinase is a major player in cellular responses to oxidative stress, p66 Shc may increase Jun kinase activity...particularly in response to exogenous oxidative stress;

ii) cellular functions important for the neoplastic phenotype. To the extent that p66 Shc inhibits signaling to MAP kinases, c-fos and Myc, we would expect p66 Shc re-expression to inhibit DNA synthesis and proliferation of the breast cancer cells. Additionally, re-expression of p66 Shc might severely impair the ability of the breast cancer cells to avert apoptotic death in the face of oxidative stress from the constant growth factor-receptor signaling, or in response to exogenous sources of oxidative stress. Further, the Shc proteins, especially p66 Shc, also have been implicated in cellular interactions with the extracellular matrix...collagen in particular. Thus, p66 Shc re-expression may alter cellular adhesion or migration on certain substrata;

iii) the growth and metastasis of breast cancers in tumor xenografts. Reduced DNA synthesis, increased propensity to apoptotic death and altered interactions with extracellular matrix may profoundly inhibit tumor growth and alter metastatic ability of cells that re-express p66 Shc.

## BODY

The body of this progress report is presented in sections preceded by the original Objectives which appear in italics. Some of the work has been submitted as an Abstract (attached in the Appendix) to the Orlando DOD Breast cancer meeting.

### *Preliminary Objective: To construct breast cancer cell lines that have been engineered to re-express the 66-kDa Shc protein.*

We transfected a p66-Shc construct (on the pLSXN vector, using Lipofectamine plus as a transfection reagent) containing base pair changes of the p46- and p52-Shc translation initiation start sites, into MDA-MB-453 and SKBR-3 breast cancer cell lines. Clones exhibiting G418 resistance(antibiotic selection marker) were isolated and tested for stable expression of the recombinant p66-Shc (Fig. 1).

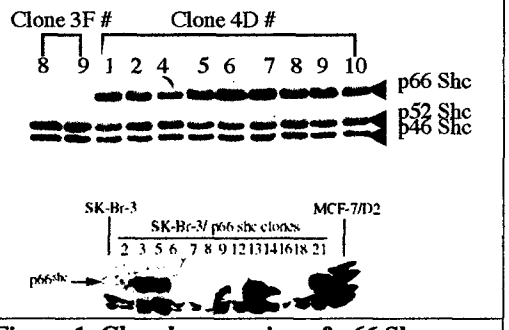


**Figure 1. Clonal expression of p66 Shc.** Levels of p66-Shc expression in the MDA-MB-453 (top panel) and SKBR-3 (bottom panel) parental cell lines and in the p66-Shc transfected stable clones. MCF-7D2 expressing endogenous p66 Shc as well as an 78-kDa Gst-p52 dn-Shc fusion protein served as a positive control. Cells were extracted in detergent-containting buffer, and proteins were immunoprecipitated with antibody to Shc and immunoblotted with antibody to Shc.

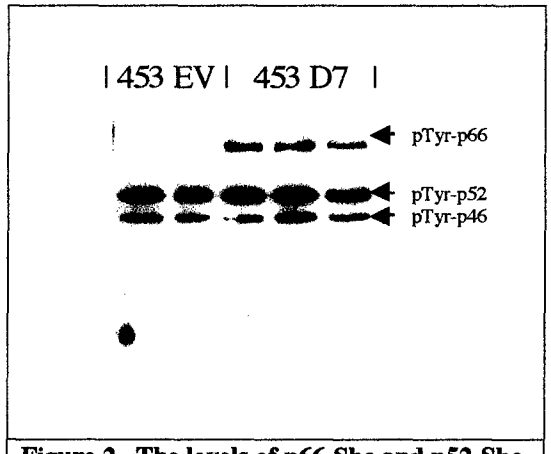


**Figure 2. The levels of p66-Shc and p52-Shc tyrosine phosphorylation are the same despite p66-Shc expression in MDA-MB-453 cells.** Proteins were immunoprecipitated as in Fig. 2 but probed with antibody to phosphotyrosine.

Several of the SKBR-3 clones expressed high levels of p66 Shc compared to the levels of expression of endogenous p46 and p52 Shc proteins. In contrast, the MDA-MB-453 sub-clone 4D7 expressed levels of p66 Shc equivalent to levels of endogenous p46 and p52 Shc. Interesingly, expression of p66 Shc in the MDA-MB-453 cells did not alter the level of p52- or p46 Shc tyrosine phosphorylation, and the p66 Shc itself became tyrosine phosphorylated (Fig. 2).

### *A major objective (originally listed as Objective iii) was to determine if forced re-expression of p66 Shc altered the growth and metastasis of the breast cancers in tumor xenografts.*

Because of difficulty in growing even the parental MDA-MB-453 and SKBR-3 breast cancer cell lines as tumor xenografts, we evaluated the effects of forced p66 Shc re-expression on their cell growth *in vitro*. Population doubling times in adherent, monolayer culture of two of the p66-

expressing clones of SKBR-3 were longer than that of parental cells or cells expressing the empty vector (Fig. 3). However, clone 13 and the MDA-MB-453 clone D7 exhibited monolayer doubling times indistinguishable from the parental cells or empty vector clones. Thus, absence of p66-Shc expression does not appear to be required for the vigorous growth of the SKBR-3 and the MDA-MB-453 breast cancer cell lines under adherent conditions in tissue culture monolayers.

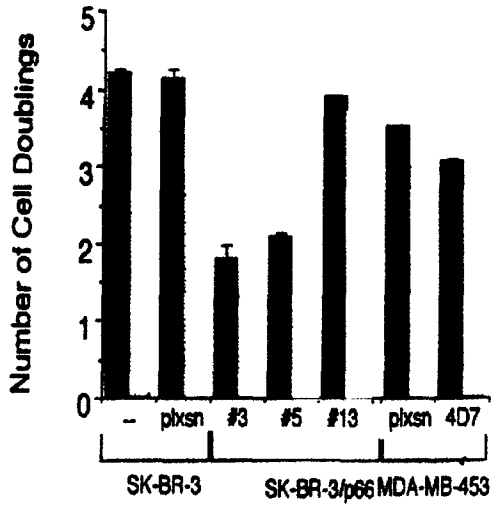


**Figure 3. The growth of SKBR-3 and MDA-MB-453 parental cells, empty vector cells and p66-Shc expressing clones in monolayer.** Cells (10,000) were seeded in triplicate into wells of 6-well plates pre-coated or not with poly HEMA, cultured 4 days, and counted.

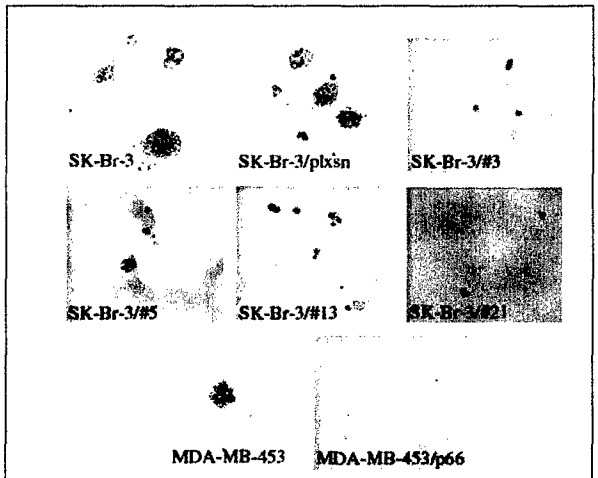


**Figure 4. Forced expression of p66-Shc inhibits colony growth on soft agar.** Breast carcinoma cell lines, SK-Br-3 and MDA-MB-453, transfected to express p66 Shc (SK-Br-3/#3, SK-Br-3/#5, SK-Br-3/#13, SK-Br-3/#21, MDA-MB-453/p66), or the pLXSN empty vector.

Perhaps the best and most widely accepted *in vitro* correlate of tumorgenicity is the ability of cancer cells, but not non-transformed cells, to grow and form colonies under anchorage-independent conditions. We asked if p66-Shc re-expression altered this critical characteristic of the breast cancer cell lines. To test this, parental, empty vector clones, and p66-Shc expressing clones of SKBR-3 and MDA-MB-453 were seeded into soft agar and colony growth was monitored. Strikingly, while parental and empty vector-containing SKBR-3 and MDA-MB-453 cells formed large, vigorous colonies, all of the p66-Shc-expressing SKBR-3 clones formed only micro-colonies, and the MDA-MB-453 clones D7 failed to grow at all (Fig. 4). Similar results were seen when cells were cultured in methyl cellulose, an anchorage-independent medium that permits easy recovery of cell populations for biochemical analyses.

The observation that p66 Shc appears to inhibit tumorgenicity (anchorage-independent growth) of the breast cancer cell lines is a major finding, and is consistent with our hypothesis that p66 Shc can function as a tumor supressor. The remaining studies sought primarily to begin to understand the mechanisms whereby p66 Shc inhibits anchorage independent growth.

First, however, we conducted preliminary investigations of the regulation of p66 Shc expression in the breast cancer cells. Pulse-chase $^{35}$S-Met metabolic labeling studies of breast cancer cells forced to reexpress p66 Shc suggested that the reduced expression of p66 Shc is not due to rapid protein

turnover...half-life of the p66 Shc protein was roughly equal to that of the p46 and p52 proteins: about 12 hrs. Furthermore, semi-quantitative PCR studies of several breast cancer cell lines suggested that the level of p66 Shc protein expression is commensurate with the levels of mRNA expression. These results suggest that the reduced levels of p66 Shc seen in breast cancers are due to regulation at an earlier level, perhaps gene transcription or mRNA stability.

***A specific objective of the proposed research was to determine the effects of p66 Shc re-expression on constitutive and growth-factor-induced signaling to MAP kinases, c-fos, Jun kinase and Myc.***

In preliminary and still ongoing experiments, we have begun to examine the effects of p66 Shc re-expression on the activity in two critical pathways possibly relevant to the mechanisms whereby p66 Shc inhibits anchorage independent growth. If p66 Shc inhibits colony formation on soft agar and methyl cellulose by inhibiting cell proliferation, we might expect it to be doing so in part by its ability to act as a feed-back down-regulator of the MAP kinases, Erk-1 and Erk-2. To examine this possibility, cells grown in monolayer culture or in methyl cellulose for 48 hrs were harvested and extracted in Laemmli sample buffer. Proteins were resolved by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antibodies that specifically recognize the activated, phosphorylated Erk proteins, and then visualized using an appropriate secondary antibody-horse-radish-peroxidase conjugate and enhanced chemiluminescence. As a control of equal loading of gel lanes, immunoblots were then stripped and re-probed with antibodies that recognized total Erk protein. The re-expressed p66 Shc does not appear to lower activity of Erk-1/-2, either under anchorage dependent or anchorage independent growth conditions (data not shown). Thus, if p66 Shc re-expression is inhibiting cell proliferation, this inhibition does not appear to be due to reduced MAPK activity. However, this needs to be confirmed, and extended to the whole two to three-weeks time course of colonial growth in methyl cellulose.

If, on the other hand, p66 Shc is inhibiting anchorage independent growth by increasing the rate of apoptotic death, we might expect p66 Shc-expressing clones to contain increased levels of activated phosphatidyl-inositol 3-kinase(PI3K). Cells under oxidative stress (from constitutive growth-factor signaling) phosphorylate serine 36 in p66 Shc. This may allow p66 Shc to bind to a complex of Grb-2-Gab-2 and PI3K. In any case, PI3K becomes activated and in turn activates AKT/PKB, which in turn phosphorylates the Forkhead transcription factor(6). The phosphorylated Forkhead cannot be translocated to the nucleus, and thus cannot stimulate transcription of its target genes, including the gene encoding catalase. Without catalase to neutralize reactive oxygen species(ROS), the cellular levels of ROS increase, ultimately inducing cell death. In a very preliminary study technically similar to that described above for activated Erks, we have observed that the p66-Shc re-expressing clones have greatly elevated levels of phosphorylated, activated AKT, compared to parental and empty vector controls. This would be consistent with p66 Shc having an impact on cell survival. Clearly, one of the first things that we'll address in our ongoing studies (supported in part by a pre-doctoral DOD fellowship...see reportable outcomes section) will be to determine if the ability of p66 Shc to inhibit colony formation is due to decreased rates of cellular proliferation, increased rates of cellular apoptosis, or both.

As one approach to elucidating the molecular pathways impacted by p66 Shc in the breast cancer

cells, we have begun to explore and identify proteins with which p66 Shc complexes. Because others have suggested that p66 Shc sequestors Grb-2, preventing or reducing productive signaling to Ras from p46 and p52-Shc-Grb-2-SOS complexes, we examined the MDA-MB-453 breast cancer cell lines for complexes of Grb2 with Shc isoforms. Although copious amounts of p66 Shc co-immunoprecipitated using antibodies to Grb2, p66 Shc expression did not diminish the amounts of p52 Shc co-immunoprecipitating with Grb2 (Fig. 5). This result is consistent with our observed inability of p66 Shc to inhibit Erk activation in these cells. We don't, however, know whether the Grb-2 is associated with p66 Shc via association with the serine(residue 36)-phosphorylated CH2 N-terminal domain of p66 Shc, via association with its phosphorylated tyrosine (residue 317), or with both.

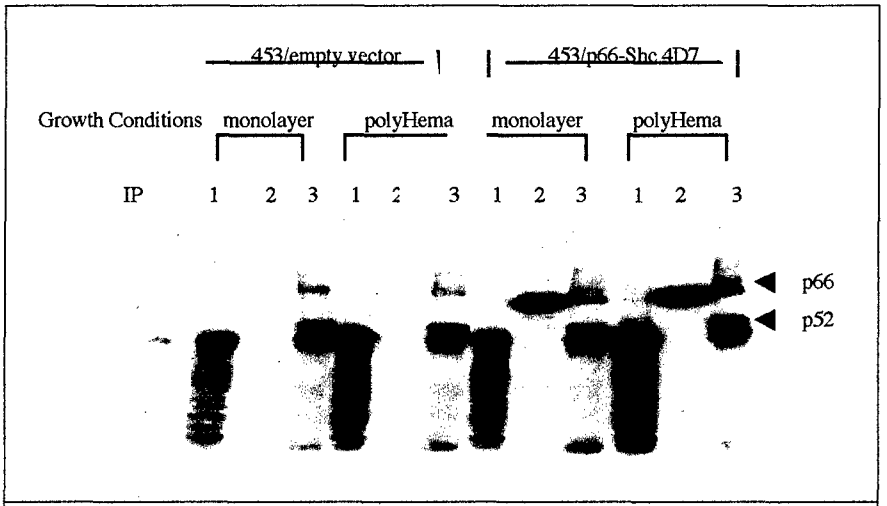


**Figure 5. Does Grb2 co-immunoprecipitate both p52- and p66-Shc?**

The parental MDA-MB-453 cells carrying the empty pLXSN vector (453/empty vector) or the clone forced to re-express p66 Shc (453/p66-Shc 4D7) were cultured for 24 hours in serum-containing IMDM media under normal anchorage dependent conditions (monolayer) or under semi-anchorage independent conditions on polyHema-coated Petri dishes(polyHema). Proteins were extracted from the cells in a buffer containing 1% Triton-X-100, and then immunoprecitated with Protein A-Sepharose 4BCL beads(negative control, lanes 1), with monoclonal antibody to p66 Shc (lanes 2) or with antibody to Grb2 (lanes 3). The immunoprecipitated proteins were resolved by 12.5% SDS PAGE, transferred to a nitrocellulose membrane, probed with a pan-Shc polyclonal antibody, and detected by ECL.

We further sought to determine if other molecules were complexed with p66 Shc, and in particular if any were complexed with p66 Shc that were not complexed with the other Shc isoforms. To address this question, MDA-MB-453 cells expressing either the empty vector or p66 Shc (clone D7) were metabolically labeled with $^{35}$S-Met, proteins were extracted in detergent and immunoprecipitated as detailed in Fig. 6. Although difficult to interpret, the re-expressed p66 Shc appears to be complexed with proteins of 22-, 28-, 45-, 50-, 85-, and 105-kDa proteins. Curiously, the pan-Shc-specific rabbit antibody immunoprecipitates the 29-kDa Grb-2 very poorly, although the p66 Shc-specific monoclonal antibodies directed to the N-terminal CH2 domain unique to p66 Shc are potent co-precipitators of Grb-2. This, taken together with the observations in Fig. 5, suggests that the epitope(s) recognized by the pan-specific Shc antibody are partially hidden by Grb-2. Similarly, the pan-Shc antibody fails to immunoprecipitate a prominent 50-kDa protein that is immunoprecipitated by the monoclonal p66 Shc antibody. This suggests that the binding of the 50-kDa protein to Shc similarly hides epitope(s) recognized by the pan-Shc antibody.

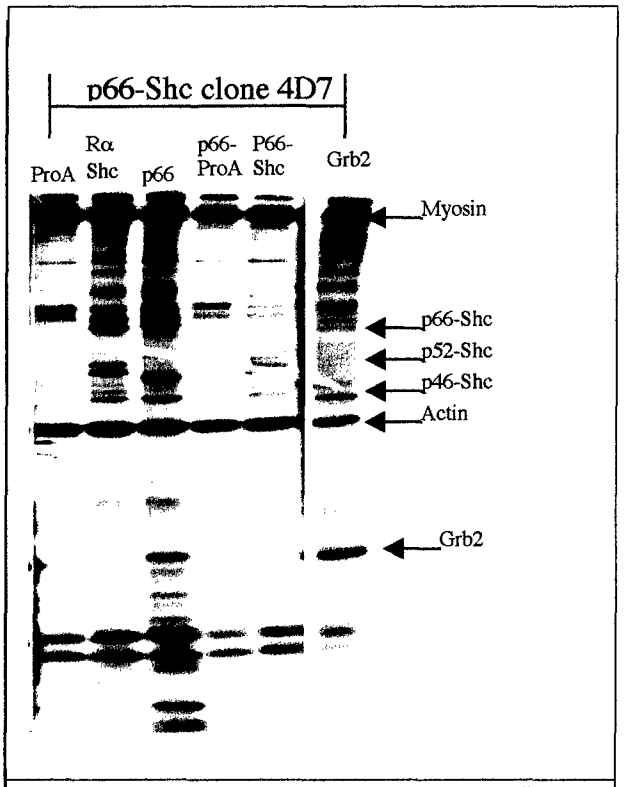


**Figure 6. p66-Shc binding proteins.** MDA-MB-453 cells re-expressing p66-Shc and the empty vector were cultured in 1 mci $^{35}S$ for four hours. Cells were then extracted with a detergent containing phosphatase inhibitors and protein kinase stimulators. Subconfluent cells were then immunoprecipitated with protein A as a negative control (ProA), with pan-Shc antibody (RαShc monoclonal antibody to p66Shc (p66), antibody to Grb2 (Grb2), cleared of p66 Shc and its complexes by p66-Sh MoAb followed by ProA (p66-ProA), and p66-Shc MoA followed by pan-Shc (P66-Shc) to detect proteins bound to p46 and p52 Shc. Proteins were resolved on a 6%-12.5% gradient SDS-PAGE gel and viewed by audioradiagraphy.

***An additional specific objective in the proposed research was to determine the effects of p66 Shc re-expression on cellular functions important for the neoplastic phenotype: DNA synthesis, cell proliferation; apoptotic sensitivity to endogenous or exogenous oxidative stress; cell migration and cell invasion.***

As mentioned above, studies of the effects of p66 Shc re-expression on cell proliferation/DNA synthesis and apoptosis are now of paramount importance and will be performed shortly, supported in part by a pre-doctoral DOD fellowship to Lisa (Williams) Nelson. Studies on the effects of p66 Shc re-expression on cell migration and cell invasion are in progress.

## KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that forced re-expression of the 66-kDa Shc isoform in breast cancer cell lines (SKBR3 and MDA-MB-453) inhibits their tumorgenicity, as measured by colony formation on soft agar and in methyl cellulose.

- Demonstration that the re-expressed p66 Shc protein complexes with Grb-2 but does not lessen Grb-2 association with the 52-kDa Shc isoform nor lessen the level of Erk activation.

## REPORTABLE OUTCOMES:

- We have developed transfected cell lines derived from the breast cancer cell lines, SKBR-3 and MDA-MB-453, that stabily and constitutively express the 66-kDa Shc isoform.

- An abstract entitled, "The 66-kDa Shc Protein Inhibits Breast Cancer Tumorgenicity", authored by Lisa R. Williams, Pamela A. Davol, and A. Raymond Frackelton, Jr. Ph.D, has been submitted for presentation at the Orlando DOD Breast meeting in September.

- Ms. Lisa Williams (now known as Mrs. Lisa (Williams) Nelson) has been awarded a pre-doctoral fellowship, BC011332, entitled, "Mechanism by which p66 Shc suppresses breast cancer tumorigenicity"

## CONCLUSIONS:

We had observed that breast cancer cell lines that harbored activated growth-factor signaling pathways displayed reduced levels of p66 Shc. Because of known growth inhibitory and apoptosis-promoting functions of p66 Shc, we hypothesized that p66 Shc acted as a tumor suppressor for such breast cancers. To begin to test this hypothesis, we have forced breast cancer cell lines to re-express p66 Shc. Cell lines re-expressing p66 Shc are unable to grow under anchorage-independent conditions, a classical correlate of tumorgenicity. Thus, p66 Shc may be a novel tumor suppressor for breast cancers.

## REFERENCES CITED:

1. Dickson, R. B., and M. E. Lippman. Growth factors in breast cancer. Endocr Rev 16:559-89,1995.

2. Stevenson LA, and Frackelton AR Jr. Constitutively tyrosine phosphorylated p52 Shc in breast cancer cells: Correlation with ErbB2 and p66 Shc expression. Breast Cancer Res & Treat, 49:119-128, 1998.

3. Stevenson, L., Ravichandran, K.S. and Frackelton, A.R.Jr. Shc dominant negative disrupts cell cycle progression in both G0/G1 and G2/M of ErbB2 positive breast cancer cells. Cell Growth & Differentiation 10:61-71, 1999.

4. Migliaccio E., et al ... Pelicci PG. Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. EMBO J. 16(4):706-716, 1997.

5. Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L, Pelicci PG. The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature 402(6759):309-313, 1999.

6. Nemoto, S. and Finkel, T. Redox regulation of Forkhead proteins through a p66shc-dependent signaling pathway. Science 295: 2450-2452, 2002.

**Individuals receiving salary support from this Award:**

A. Raymond Frackelton, Jr., Principal Investigator
Pamela A. Davol, Research Associate

# APPENDIX

## The 66-kDa SHC PROTEIN INHIBITS BREAST CANCER TUMORGENICITY

**Lisa R. Williams, Pamela A. Davol, and A. Raymond Frackelton, Jr. Ph.D.**

Departments of Medicine and Pathology, Roger Williams Medical Center and Brown University, 185 Chalkstone Avenue, Providence, RI 02908

Email: Lisa_Williams@Brown.edu

Poor prognosis in breast cancer is often associated with constitutive activation of ErbB2, EGF, IGF-1 or other cellular receptors. Most breast cancer cell lines have high levels of activation (tyrosine phosphorylation) of a downstream signaling molecule, the 52-kDa Shc adapter protein, central to all of these signaling pathways and implicated in driving cell motility and proliferation. In contrast, a 66-kDa form of the Shc protein that appears to function normally as a feedback inhibitor of growth-factor signaling was absent or present at reduced levels in these same cells. In a companion abstract in these proceedings (Frackelton et al.), we report that increased levels of tyrosine phosphorylated Shc coincident with decreased expression of p66 Shc serve as an excellent predictor of aggressive breast cancers with high likelihood of disease recurrence.

The purpose of the research under this Concept Award was to begin to explore the regulation of the p66 Shc isoform and to determine if its reduced expression in breast cancers was required for some critical aspect of the neoplastic phenotype. Pulse-chase $^{35}$S-Met metabolic labeling studies of breast cancer cells forced to reexpress p66 Shc suggest that the reduced expression of p66 Shc is not due to rapid protein turnover. Furthermore, semi-quantitative PCR studies suggest that the level of p66 Shc protein expression is commensurate with the levels of mRNA expression. These results suggest that the reduced levels of p66 Shc seen in breast cancers are due to regulation at an earlier level, perhaps gene transcription, splicing or mRNA stability.

To test the role of the p66 Shc splicing isoform on the neoplastic phenotype, the SK-BR-3 and MDA-MB-453 cell lines were forced to re-express p66-Shc by stabily transfecting them with a pLXSN vector containing the full length p66 Shc coding region. After G418 selection, multiple clones were isolated that stabily expressed p66-Shc at levels comparable to non-transformed breast epithelial cells. The p66 Shc-expressing SK-BR-3 and MDA-MB-453 clones showed no obvious or consistent differences from the parental lines or from clones carrying the pLXN empty vector in their morphology or growth rates under adherent conditions. In contrast, however, the p66-Shc expressing clones failed to form colonies when grown under anchorage-independent conditions in soft agar or methyl cellulose. We are currently exploring the molecular requirements and molecular mechanisms whereby the 66-kDa Shc isoform inhibits this *in vitro* correlate of tumorgenicity, anchorage independent growth.

Reduced expression of the 66-kDa Shc isoform predicts poor prognosis in node-negative breast cancer. Furthermore, in vitro, its reduced expression appears to be required for tumorgenicity. Understanding the mechanisms by which p66; Shc inhibits breast cancer tumorgenicity will allow us to better understand its role in the development of breast cancer, and should help us to the better design of rationale, targeted therapies.

Supported by the U.S. Army Medical Research and Material Command under DAMD17-01-1-0617.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 10 Jun 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB270849
ADB263653
ADB282255
ADB262232
ADB277418
ADB285664
ADB281628
ADB284965
ADB265796
ADB282152
ADB285670
ADB257200
ADB266081
ADB285703
ADB285699
ADB285481
ADB285668
ADB283633